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Determination of the Site(s) of Action of Selected Pesticides by an Enzymatic-Immunobiological Approach

Mississippi State Univ, Mississippi State Dept of Biochemistry

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16. ABSTRACT

This report describes development of an antibody to an organochlorine pesticide to be used in studies related to its inhibition of the ATPase system. Kelevan, the condensation product of ethyl levulinate and Kepone, was successfully conjugated to bovine serum albumin (BSA), fibrinogen (BF), and gamma globulin (BGG). Rabbits and chickens preimmunized with BSA and then immunized with BSA-Kelevan produced antibodies to both the hapten, Kelevan, and the carrier protein BSA. Antiserum to Kelevan protected ATPase activity against Kepone and its derivatives. The titer of antibody to Kelevan was critical since antiserum with only trace amounts of Kelevan antibody failed to protect the ATPase activity against Kepone Inhibition. Antibody was concentrated by Na_2SO_4 fractional precipitation of the antiserum and obtained in pure form by affinity chromatography using BGG-Kel covalently linked to Sepharose 4B. Pure antibody was obtained from untreated blood serum or plasma with no prior pretreatment or fractionation using the BGG-Kel affinity column. Complete protection of mitochondrial mg^{2+}ATPase activity from *in vitro* inhibition of Kepone was obtained using a 1.2 mg quantity of na_2SO_4 fractionated antibody and only 120 µg of pure antibody. Reversal of ATPase inhibition was readily obtained by addition of antibody prior to addition of substrate to the reaction mixture.

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DETERMINATION OF THE SITES(S) OF ACTION OF SELECTED PESTICIDES BY AN ENZYMATIC-IMMUNOBIOLOGICAL APPROACH

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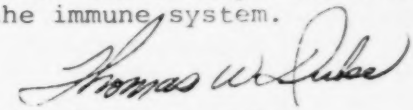
FOREWORD

The protection of our estuarine and coastal areas from damage caused by toxic organic pollutants requires that regulations restricting the introduction of these compounds into the environment be formulated on a sound scientific basis. Accurate information describing dose-response relationships for organisms and ecosystems under varying conditions is required. The Environmental Research Laboratory, Gulf Breeze, contributes to this information through research programs aimed at determining:

- the effects of toxic organic pollutants on individual species and communities or organisms;
- the effects of toxic organics on ecosystem processes and components;
- the significance of chemical carcinogens in the estuarine and marine environments.

In understanding of toxicological processes it is of utmost importance that the mechanisms responsible be known. This report deals with one of these processes: the response of an organism to a compound recognized as foreign by the immune system. While this mechanism is probably of little consequence in acute poisoning, compounds such as DDE with long residence times in the blood stream may ultimately be deactivated through the possible action of antibodies formed as a response to the toxicant. As work of this type continues, there should be an answer as to whether or not the already established detoxification/excretion processes or elimination of toxic compounds from an organism can be supplemented by the immune system.



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Director
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ABSTRACT

This research program was initiated with the overall objective of producing an antibody to an organochlorine pesticide which would be used in studies on its mechanism of inhibition of the ATPase system from various sources. A single pesticide (Kelevan) was chosen for use as a hapten for covalent conjugation to various protein antigens. The approach to this study included organic synthesis and instrumental analysis, antibody production and detection by immunological procedures, and biochemical investigations to determine the effects of antibody on the inhibition of the ATPase system by Kepone, a derivative of the hapten, Kelevan.

Kelevan, the condensation product of ethyl levulinate and Kepone, was successfully conjugated to bovine serum albumin (BSA), fibrinogen (BF), and gamma globulin (BGG). Rabbits and chickens preimmunized with BSA and then immunized with BSA-Kelevan produced antibodies to both the hapten, Kelevan, and the carrier protein BSA. Antiserum to Kelevan protected ATPase activity against Kepone and its derivatives. The titer of antibody to Kelevan was critical since antiserum with only trace amounts of Kelevan antibody failed to protect the ATPase activity against Kepone inhibition.

Antibody was concentrated by Na_2SO_4 fractional precipitation of the antiserum and obtained in pure form by affinity chromatography using BGG-Kel covalently linked to Sepharose 4B. Pure antibody was obtained from untreated blood serum or plasma with no prior pretreatment or fractionation using the BGG-Kel affinity column. Complete protection of mitochondrial Mg^{2+}ATPase activity from *in vitro* inhibition of Kepone was obtained using a 1.2 mg quantity of Na_2SO_4 fractionated antibody and only 120 µg of pure antibody. Reversal of ATPase inhibition was readily obtained by addition of antibody prior to addition of substrate to the reaction mixture. In a brief study conducted after the completion date of this grant, it was observed that the antibody to Kelevan was not specific for Kepone but also prevented inhibition by other chlorinated hydrocarbon pesticides. The possible meaning of this observation is briefly discussed.

This report was submitted in fulfillment of Grant No. R803458 by Mississippi Agricultural and Forestry Experiment Station, Mississippi State University under the partial

sponsorship of the U. S. Environmental Protection Agency. This report covers the period from February 15, 1975 to February 14, 1978; work was completed as of April 28, 1978.

CONTENTS

Foreword iii

Abstract iv

List of Abbreviations vii

Introduction 1

Conclusions and Recommendations 3

Chemistry Studies 5
Materials and Methods 5
Results and Discussion 7

Immunology Studies 11
Materials and Methods 11
Results and Discussion 13

Biochemistry Studies 16
Materials and Methods 16
Results and Discussion 17

References 27

LIST OF ABBREVIATIONS

Kepone (Kep)	--[1,2,3,4,6,7,8,9,10,10-decachloro-pentacyclo $(5.3.0.0^{2,6}.0^{3,9}.0^{4,8})$-decan-5.5 dihydroxide]
Kelevan (Kel)	--[ethy] δ-(-5-hydroxyl-1,2,3,4,6,7,8,9,10,10-decachloro-pentacyclo-$\{5.3.0.0^{2,6}.0^{3,9}.0^{4,8}\}$ decyl) levulinate].
Reduced Kepone (DCPD)	--[1,2,3,4,6,7,8,9,10,10-decachloro-pentacyclo $(5.3.0.0^{2,6}.0^{3,9}.0^{4,8})$-decan-5-ol].
BSA	--Bovine serum albumin
BGG	--Bovine gamma globulin
BF	-- bovine fibrinogen
KS	--Kelevan N-hydroxysuccinimide

INTRODUCTION

During studies on the ATPase systems, Koch (1964, 1969/70) observed and reported sensitivity to organochlorine pesticides at micromolar concentrations. He suggested that inhibition of the ATPase enzymes by organochlorine pesticides could be associated with the mechanism of toxic action of these compounds. Work has continued in Koch's laboratories in conjunction with a number of colleagues but especially in the laboratory of L. K. Cutkomp and with the excellent collaboration of D. Desaiah. Of the over thirty papers published by these authors on this topic of ATPase sensitivity of organochlorine pesticides, the following selected references strongly indicate the relationship between ATPase inhibition and mechanism of toxic action (Desaiah and Koch, 1975; Desaiah, et al., 1975; Cheng and Cutkomp, manuscript submitted; Koch et al., 1977; other references by these authors are given in these papers).

Also in vivo studies have been reported which indicate that mitochondrial Mg^{2+}ATPase inhibition occurred in fish brain and rat liver. In an in vivo study on DDT inhibition of ATPase activity in fish Pimephales promelas, specific inhibition of mitochondrial (Mito) Mg^{2+}ATPase activity in brain was observed (Desaiah et al. 1975). Although a number of exposed fish died prior to completion of the study, the maximum inhibition observed for this enzyme activity in the surviving fish after 266 days exposure was only about 60%. Therefore, the 50-60% inhibition of brain oligomycin sensitive (Mito) Mg^{2+}ATPase activity was considered sufficient to cause death, or a partial reactivation of the enzyme activity occurred during homogenization and fractionation procedures. A similar phenomenon was also observed in liver preparation of rats fed with Kepone for 16 days (Desaiah et al., 1977).

To obtain more definitive information on their mode of action, it was proposed to produce an antibody to an organochlorine pesticide. The antibody with high affinity for its specific antigen would be used to gain insight into the nature of the interaction of the enzyme complex with the pesticide. This final report presents the results of a three-year study on this project under partial support by EPA Grant R803458 (from February 15, 1975 to February 14, 1978).

The use of pesticides as haptens for antibody production has been reported by Anderson et al., (1964), Langone and Van

Vunakis (1975), Cento et al. (1970), and Haas and Guardia (1968). The high specificity of antibodies for haptens (in this case pesticides conjugated to antigenic protein) have caused interest in their use for the development of highly sensitive radioimmunoassay procedures for pesticides (Langone and Von Vunakis, 1975).

In studies on the mode of action of organochlorine pesticides, it was shown that Kepone and its monohydroxy-reduced derivative strongly inhibited ATPase system in fish brain preparations (Desaiah and Koch, 1975a). Further, because of the availability of a highly purified derivative of Kepone, Kelevan (an analytical standard grade), it was proposed to use Kelevan as the hapten to be covalently linked to the antigen BSA.

This final report is divided into three parts according to the discipline required to conduct the three different aspects of the research program: I Chemistry Studies; II Immumology Studies; and III Biochemistry Studies.

CONCLUSIONS AND RECOMMENDATIONS

An antibody to the pesticide Kepone was produced in rabbits and chickens. The antigen-hapten complex (BSA-Kelevan) used for stimulation of antibody production was synthesized by reacting BSA with the succinimide derivative of Kelevan. In addition, Kelevan derivatives were prepared using BGG and BF following the same procedures. The latter two derivatives were used for the detection of antibody in blood serum by the micro-ouchterlony immunodiffusion test.

An affinity column was prepared by covalently linking BGG-Kel to a cyanogen bromide activated Sephrose 4B support matrix. Purified antibody was prepared by passing blood serum directly through the column. The affinity bound antibody was removed by 2M KCl buffer solution. Antibody obtained from the affinity column was shown to be highly active in preventing or reversing inhibition by Kepone. An unexpected result was observed in that the antibody also protected against other organochlorine pesticides (DDT and aldrin). However, the antibody did not reverse the inhibition by the pesticide Plictran (a non-chlorinated compound).

It is concluded from the latter result that the antibody exerts its action on organochlorine type structures rather than the particular compound which was used as the hapten. This result may indicate that inhibition of the ATPase system by organochlorine pesticides occurs through a similar mechanism, such as disruption of secondary bonding forces of the lipo-protein complex of the enzyme system.

It is recommended that further studies be conducted using the antibody Kelevan to determine its effect on inhibition by other organochlorine pesticides and using different tissue sources for the ATPase system. It is also recommended that at least one additional antibody to an organochlorine pesticide (such as DDT or Kelthane) be produced to compare the effects of such an antibody on the inhibition by Kepone and other organochlorine pesticides. If the two antibodies have similar effects, this would indicate that the antibody's specific binding site is not just directed toward a particular compound but also directed towards a type of structure in that compound. Antibody cross-reactivity by similar structures is not surprising, but the molecular structures of Kepone and DDT would appear to be quite different. However, molecular modeling of the

two compounds to observe for 3-dimensional configurations could prove to be important. Such information, if this could be obtained from the above studies, might yield information concerning why compounds like DDT and Kepone exert different intensities of inhibition of the ATPase system; namely, DDT is more effective on mitochondrial Mg^{2+}ATPase than Kepone.

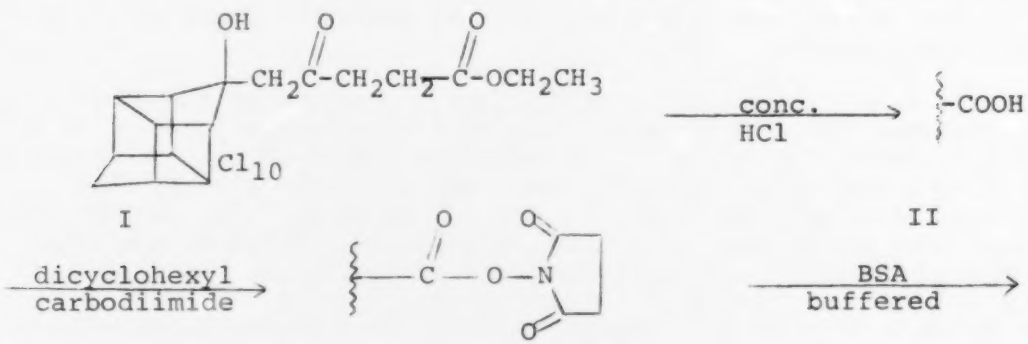
It is also recommended that studies be conducted on experimental animals exposed under in vivo conditions to Kepone (or other selected pesticides) to determine whether there is a "target" tissue for the pesticide. These studies would include removal of tissue after in vivo exposure to sublethal concentrations of Kepone for varying periods of time. The tissues would be homogenized, fractionated and ATPase activity determined, followed by measurement of reversal of ATPase activity by exposure to the pure antibody to Kepone. The tissue showing the greatest reversal of inhibition would be the most sensitive site of action of the pesticide. This information would be useful in designing stress or behavioral tests which would indicate reduced physiological capacity due to pesticide toxicity.

CHEMISTRY STUDIES

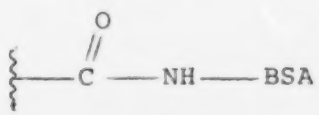
Materials and Methods

Conjugation of Kelevan to Bovine Serum Albumin (BSA)

The procedure chosen to functionalize the hapten and to conjugate it to BSA is as follows:



III



IV

Kelevan (I) [ethyl δ-(5-hydroxy-1,2,3,4,6,7,8,9,10,10-decachloropentacyclo{5.3.0.$0^{2,6}$0.^{3,9}0.^{4,8}}decyl) levulinate] was first hydrolyzed to the free acid (II) with concentrated hydrochloric acid. The free acid was then treated with N-hydroxysuccinimide to produce the ester (III). This type of ester has previously been shown to rapidly react with the amine groups of amino acids to yield amides (Anderson et al., 1964) and with free amine groups of protein to yield hapten-protein conjugates (Langone and Van Vunakis, 1975). Therefore, treatment of III with BSA was expected to produce the desired conjugate (IV) by forming amide bonds with the free groups of the lysine units of BSA. Kelevan was supplied by the Allied Chemical Corporation. All other reagents and solvents were purchased from commercial

sources. Melting points were determined on a Mel-Temp apparatus and are uncorrected while infrared spectra were determined on a Perkin-Elmer Infracord in KBr wafers.

Functionalized Hapten

To a solution of Kelevan (1) (52.9 g; 0.1 mole) in 50 ml of ether was added 150 ml of concentrated hydrochloric acid. The resulting solution was heated to reflux for 6 hr. The solution was concentrated until the free acid separated as an oil. Purification of the crude product was achieved by dissolving the oil in ether, drying the solution with molecular sieve (8-12 mesh), and removing the solvent. By this procedure 39 g (78%) of purified acid was obtained [infrared absorption for C-OH at 3400 cm^{-1} was strong and broad and for the carboxyl group at 1700 cm^{-1} was strong].

To a cooled solution containing a mixture of the free acid of Kelevan (39 g; 0.78 mole) and of N-hydroxysuccinimide (8.97 g; 0.78 mole) in 250 ml of dioxane was added 15.9 g (0.78 mole) of n, N-dicyclohexylcarbodimide. The reaction mixture was allowed to stand overnight in the refrigerator. The precipitated dicyclohexylurea was collected, washed with dioxane, and the combined filtrate and washings were concentrated until all of the solvent was removed. Infrared analysis of the residue showed it to be a mixture of desired derivatives and dicyclohexylurea. Repeated crystallizations of the mixture from methylene chloride and 1-propanol gave the desired derivative as colorless crystals, m.p. 245°, infrared absorptions for C=O at 1700, 1740, 1790, and 1830 cm^{-1} and for C-OH at 3340 cm^{-1}.

Conjugation of Functionalized Hapten for Immunization

To a solution of 100 mg of BSA in 100 ml of water (buffered to pH 7.1) was added 114 mg of functionalized hapten dissolved in 5 ml of tetrahydrofuran. (Previous control experiments indicated this amount of tetrahydrofuran [THF] would not denature the protein). The mixture was then stirred and cooled for 30 hr. The Kelevan-protein derivative containing mixture was concentrated by freeze-drying. The residue was redissolved in water or 0.1M NaCl, passed through a Sephadex G-25 column and the 280 nm peak from the column was collected and freeze dried. The 280 nm absorbance indicated separation of the total protein from the other components in the conjugation reaction. The freeze dried preparations were analyzed for Kelevan and used for immunology studies.

Analysis of the Kelevan-BSA Conjugate

A method for analysis of Kelevan in protein samples was developed. This method had high sensitivity (microgram levels) and was capable of detecting Kelevan bound to protein. The

analysis was accomplished by oxidizing both the protein and Kelevan (which oxidizes to Kepone) and analyzing for Kepone. In an oxygen atmosphere at 400°C, Kelevan is oxidized to Kepone; whereas, the protein is oxidized to various gaseous products. A sample (1-3 mg) was placed in a 25 ml ampule. The ampule was evacuated to 0.5 mm of mercury pressure, filled with oxygen (160 mm of mercury pressure), and then sealed. The sealed ampule was placed in an oven (400°C) for five minutes and then removed and allowed to cool. After the cooled ampule was opened, the Kepone was extracted by washing the ampule several times with methanol and petroleum ether. The extracts were combined, diluted to a standard volume, and analyzed for Kepone on a gas chromatograph equipped with an electron capture detector.

The concentration of Kelevan initially present in the sample was calculated from the amount of Kepone found after oxidation. The efficiency for oxidizing Kelevan to Kepone (approximately 30%) was determined for each set of samples by including one sample which contained the protein (1-2 mg) and Kelevan (1-2 mg).

Preparation of a Kelevan-Bovine Gamma Globulin Conjugate for Specificity Studies

In order to determine if the antibody for Kelevan-BSA was specific for Kelevan, Kelevan-BGG and Kelevan-BF were prepared. The procedure employed for conjugation was identical with that used for preparing the Kelevan-BSA conjugate.

Results and Discussion

Pure Kelevan was available as analytical standard. It was selected as a Kepone derivative for conjugation to protein because the levulinate group of Kelevan could act as a means of extending the hapten groups from the large protein molecule. The use of such a "bridge" is common to enhance the antibody production to the hapten (in this study the hapten is related to Kepone and to the 5-monohydroxy reduced derivative of Kepone).

Conjugation of Kelevan to BSA by the procedure outlined in the Materials and Methods, i.e., adding functionalized hapten in a THF solution and purification of the conjugate by freeze-drying and Sephadex G-25 chromatographic separation, yielded the sample for immunization studies. Figure 1 and Table 1 give an example of the 280 nm absorbance curve and Kelevan content of fractions from G-25 gel filtration.

Analysis of several Kelevan-BSA conjugates as described in the Materials section gave values of Kelevan in the samples of 2.5 ± 0.5%. These results are the average of three runs. In

earlier preparations, other sets of duplicate runs gave values as high as 15% Kelevan (probably due to contamination by uncombined Kelevan) in the conjugate and as low as 0%. To trace the source of the variation in the results, several standard runs were made in which various parameters were changed. The variables in the results were found to be due to any remaining imidazole (used as a buffer in the synthetic step) in the samples which had been purified by Sephadex G-25 column chromatography and to the time allowed for the conjugation reaction. If known amounts of Kelevan and imidazole are placed in a sealed ampule and treated by the procedure described for analysis, no Kelevan is found by g.c. analysis. Apparently, imidazole totally dechlorinates the Kelevan and, therefore, the electron-capture detector on the g.c sees no product. This could mean that the 2.5 ± 0.5% values for Kelevan in the conjugate were minimum values. Because of these results, the buffer used for the conjugation reaction was changed to 0.1M sodium phosphate pH 7.1. Yield of BSA-Kel was slightly reduced but problems with Kelevan analysis were eliminated.

Results of analysis of preparations made during 1977 using phosphate buffer are given in Table 1. Only preparations which showed the presence of approximately 2.5% Kelevan conjugated to BSA were used for injection in animals for antibody production.

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TABLE 1. KELEVAN CONCENTRATION (%) IN VARIOUS PROTEIN-KELEVAN CONJUGATES

BSA-Kel		BGG-Kel**		BF-Kel**	
Tube #	Kelevan	Tube #	Kelevan	Tube #	Kelevan
3-5	2.6%	2-3	0.1%	2-3	0.1%
6	3.4%	4-6	0.4%	4-6	0.5%
7	0.5%				
8-12	0.02%	7-9	0.7%	7-10	0.25%
13-15	0.1%				
19-27	55.0%*				

*Nonconjugated Kelevan.

**Fractionation not considered satisfactory because considerable material was lost due to insolubility of protein due to concentration of solutions from chromatographic separation. Therefore, BGG-Kel and BF-Kel were purified by extensive dialysis against distilled water. The resulting products contained 1.1% Kel (BGG-Kel) and 2.4% Kel (BF-Kel) some of which may have been non-covalently bound.

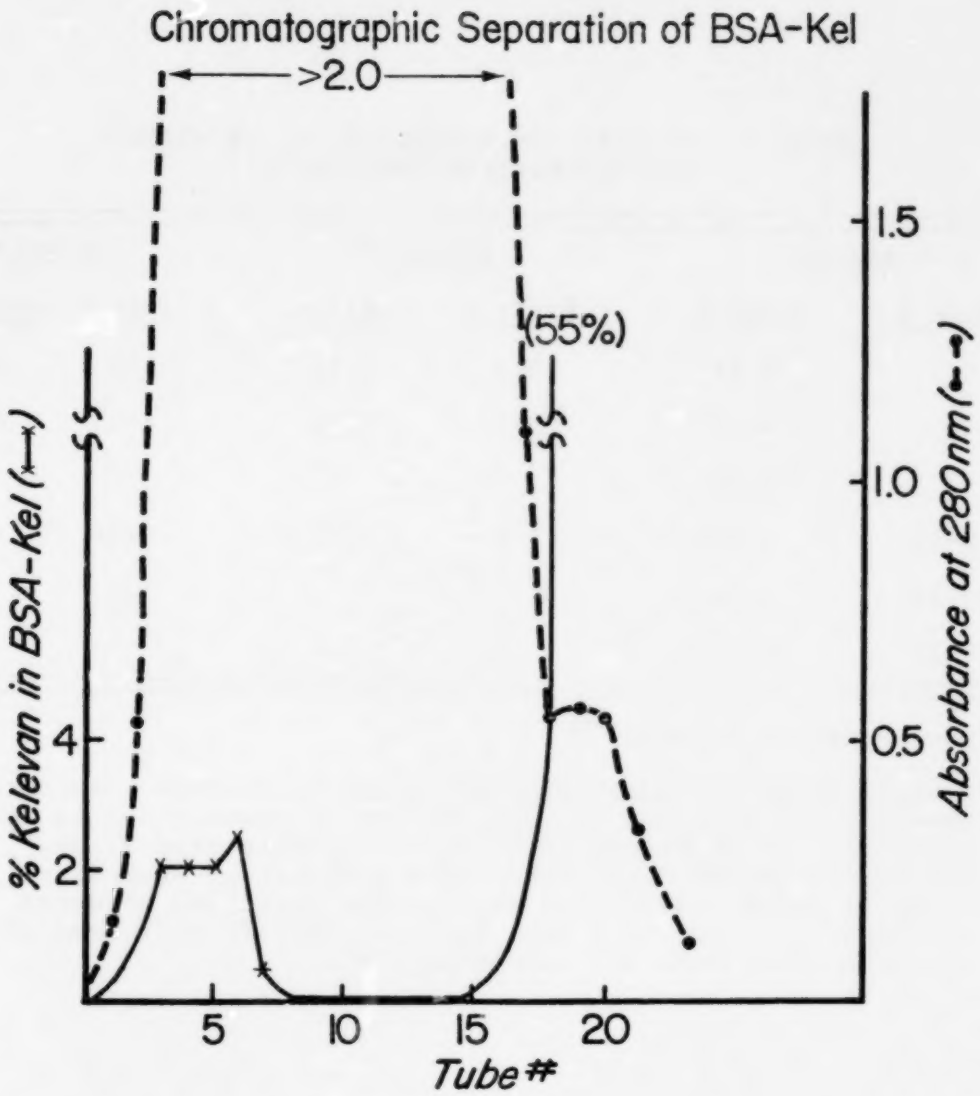


Figure 1. Separation of components in BSA-Kelevan conjugation reaction mixture. (THF removed on roto-evaporator; mixture centrifuged 18,000 x g for 10 min.; supernatant concentrated by freeze drying; solubles from thawed samples separated by using Sephadex G-25 chromatographic column (1.5 x 30 cm) using distilled water or 0.1M NaCl as eluent; 5 to 7 ml fractions collected). Tube 3-6 had a distinct yellowish color not present in the other tubes (see Table 1). Tubes 19-23 combined and the high concentration of Kelevan to BSA (55%) indicated the presence of unbound Kelevan.

IMMUNOLOGY STUDIES

Materials and Methods

Antiserum Preparation

Three rabbits and fifteen New Hampshire chickens were primed with soluble bovine serum albumin (BSA) at week zero. One week later, the rabbits were supplementally immunized in the footpads with a complete Freund's adjuvant (CFA) emulsion containing 5 mg/ml BSA-Kel conjugate. All rabbits were injected subsequently in weeks 6, 9, and 16.

Blood was collected from the marginal ear vein from both control and experimental rabbits in weeks 7, 11, and 18. The blood was allowed to clot at room temperature for 1 hour, the clot ringed, and the serum allowed to separate overnight at 4°C. The serum was centrifuged (10 minutes at 1200 rev/min) and stored at 4°C.

A slightly modified procedure was employed with the chicken. One week after the primary injection, the chickens were supplementally immunized intramuscularly with CFA emulsion containing 5 mg/ml BSA-Kel conjugate. All chickens were injected subsequently in weeks 6, 9, and 16.

Blood was collected by cardiac puncture from both control and experimental birds in weeks 7, 11, and 18. The blood was allowed to clot for 1 hour, the clot ringed, and the serum allowed to separate overnight at room temperature. The serum was centrifuged (10 minutes at 1200 rev/min) and stored at 4°C.

Both the rabbits and chickens were immunized using an adjuvant modified antigen. Adjuvants can be described as substances that when mixed with an antigen prior to injection, enhance antibody production. They are particularly useful if the antigen is of low immunogenicity. Freund's complete adjuvant is a water-in-oil emulsion, composed of antigen in saline and a mixture of an emulsifier with Mycobacteria (Campbell et al., 1970).

In the last year of the grant, we utilized the chicken more extensively than the rabbit because chickens are better producers of precipitating antibody than are rabbits (Wolfe et al., 1942, 1957, 1960). For example, it has been shown that rabbits produce

only microgram amount of precipitating antibody protein per milliliter of serum against human hemoglobin after extensive injections, whereas chickens produce as much as 3 mg of precipitating antibody protein per milliliter of serum after only 4 intraperitoneal injections of the same antigen (Goodman and Campbell, 1953).

Ouchterlony Double-Diffusion (Theory)

In double diffusion, antigen and antibody migrate toward each other through gel which originally did not contain either of these reagents. As the reagents come in contact with each other, they combine to form a precipitate that is trapped in the gel matrix and immobilized. This test offers the unique advantage of not only enumerating the minimum number of antigen-antibody systems reacting in a given mixture, but also indicating the relationship among various antigens. This type of test depends on the specific precipitation of antigen-antibody complexes at the proper ratios and on the solubility of the precipitate in excess of antigen or antibody. Only those antigens that migrate through the gel and for which antibodies are present in sufficient concentration in the antiserum can form lines of precipitation.

Four basic patterns can be obtained with an antiserum and preparations of single antigens.

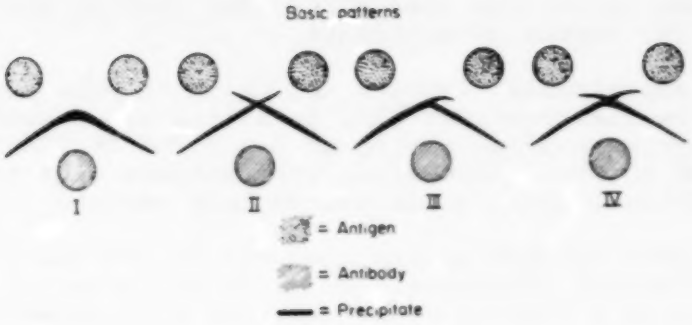
I. Pattern of identity - both antigens have a set of identical determinant groups with respect to the antiserum employed.

II. Pattern of nonidentity - antiserum does not contain antibodies to any determinant groups common to both antigens, and thus the two lines formed are completely independent of each other and cross without interaction.

III. Partial identity - one antigen (Basic Patterns III, below) reacts more fully with the antibodies employed, while the other antigen reacts with fewer antibodies, allowing formation of a "spur."

IV. Two antigens have at least one type of determinant group in common and, in addition, each has antigenic groups not shared by the other. Thus the reaction obtained is that of partial coalescence on both sides and formation of double spurs (Williams and Chase, 1971).

Depending on concentration of antibodies and antigens involved, many modifications of these four basic patterns can be obtained.



Basic precipitate patterns obtained in double gel diffusion. I. Complete coalescence. Reaction of identity. II. Absence of coalescence. No cross-reaction. III. Partial coalescence of one antigen. Cross-reaction. IV. Partial coalescence of two antigens.

Antibody Protection

A micro-adaption of Ouchterlony's technique (Auernheimer and Atchley, 1962; Brewer, Glick, and Vinson, 1972; and Glick, 1976) was utilized to determine the presence of specific antibody to Kelevan. Antigens used in the study were BSA, BSA-Kelevan, bovine gamma globulin (BGG), BGG-Kelevan, Kelevan, fibrinogen-Kelevan, and fibrinogen (FB).

The central well was charged with antisera and the peripheral wells with antigens and allowed to stand for 24-48 hr at 25°C, for maximum precipitin line development. The templates were then removed and the slides placed in physiological saline (0.9%) for 48 hr. The slides were then rinsed in distilled water for 10-15 minutes and placed in 20% acetic acid for 1 hr. The slides were stained for 90 sec in 2 ml of 0.1% Amido Schwartz 10B. They were rinsed in distilled water and allowed to air dry.

Results and Discussion

Antibody Production to Kelevan

Preimmunization of rabbits with a carrier protein prepares the animal for markedly enhanced antibody production to a second molecule conjugated to that carrier (Katz *et al.*, 1970 and Paul *et al.*, 1970). Their studies indicate that the carrier

specificity of hapten-specific anamnestic antibody responses is largely due to the interaction of two independent cell-associated recognition units, one specialized for carrier and the other specific for hapten determinants.

All experimental rabbits and chickens were preimmunized with the carrier protein, BSA, in saline and subsequently received primary immunization of 1.0 mg/ml of BSA-Kelevan emulsified in CFA. Subsequent inoculations were made according to the protocol outlined in the Methods section.

Separate antibodies are produced to BSA and to Kelevan in a hapten-carrier conjugate. An affinity column to Kelevan conjugated to a protein other than BSA will selectively remove antibody to Kelevan. When this isolated antibody was tested by the micro-Ouchterlony procedure, precipitin bands appeared where Kelevan was as an antigenic determinant regardless of the carrier molecule (i.e., BSA, BGG, or FB) [Fig. 2, Card D]. This was true whether the antibody was produced in a rabbit or chicken. On the other hand, if serum from a BSA-injected animal was reacted against the same antigens, precipitin bands developed against BSA and not against Kelevan (see Fig. 2, B).

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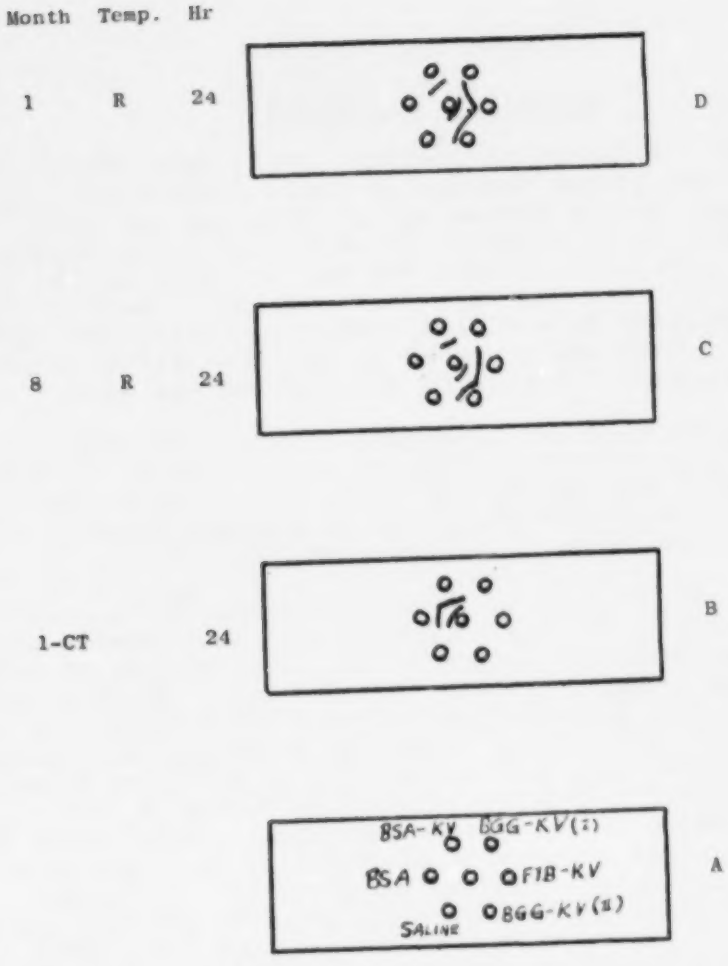

Figure 2. Micro-Ochterlony precipitin reactions of antibodies from blood serum from BSA and BSA-Kel immunized rabbits and chickens. A. Blank slide indicating the positions of various solutions antigen components. Antibody preparation was placed in the center well. B. Antibody from chicken (serum) immunized only with BSA. C. Antibody from rabbit (serum) immunized with BSA-Kel. D. Antibody from chicken (serum) immunized with BSA-Kel.

BIOCHEMISTRY STUDIES

Materials and Methods

Kepone and its reduction product, DCPD, were chosen as representative pesticides because of their broad inhibitory action (in vitro) on the ATPase system (Desaiah and Koch, 1975a) and the availability of Kelevan (as mentioned in an earlier section). The covalently linked BSA-Kel was used as the antigen for antibody production (see previous section). Kepone and Kelevan were obtained from Allied Chemical Company and were recrystallized. DCPD was prepared at the Mississippi State Chemical Laboratories. Purity of all samples was 98+%.

Serum from control and immunized rabbits was used for preparation of immunoglobulin (Ig) by the procedure of Kulkarni and Glick (1974). The second 14% (w/v) Na_2SO_4 Ig sediment (9,000 x g for 10 min) was suspended at similar protein levels (1.2 mg/ml) in 0.1M imidazole buffer, pH 7.5.

Sepharose 4B was used as support material for preparation of a BGG-Kel affinity column. Sepharose 4B was reacted with CNBr in alkaline solution according to the procedure of Axen as described by Cuatrecasas (1969). The Sepharose 4B-CN preparation was slowly stirred overnight at 4° after addition of BGG-Kel. BGG was thus covalently linked to Sepharose 4B. After washing, the complex was used to prepare an affinity column (2X30 cm) for specific binding antibodies to the hapten (Kel) produced in response to the antigen BSA-Kel. Blood serum or plasma from immunized rabbits or chickens (4 to 6 ml) were allowed to penetrate into the top of the affinity column (previously washed with 0.1mM Tris-HCl pH 7.0) and the unbound constituents were eluted with 10mM Tris-HCl pH 7.0. When the effluent from the column showed zero absorbance at 280 nm, the eluting solvent was changed to 2 M KCl in 10 mM Tris-HCl, pH 7.0. The effluent was collected in 5-7 ml fractions and the tubes containing 280 nm absorbing material were combined; and then concentrated and washed with 10 mM Tris-HCl using an XM-50 Diaflo filter in an Amicon Stirred Ultrafiltration Cell using N_2 pressure at about 10 psi.

Sources of tissue for preparation of 13,000 x g sediment (mitochondrial and nerve ending particle) of subcellular particle fractions from homogenates (in 0.32M sucrose, 10 mM Imidazole buffer pH 7.5, 1 mM EDTA), were fire ant head, dog brain cortex, housefly head and thorax, and mice and rat hearts. Homogenization

and fractionation were as reported by Koch (1969a). Protein content of each fraction was determined by the method of Lowry et al. (1951) using BSA as the standard. Samples were then diluted to contain 15 to 25 µg protein per 25 to 50 µl homogenate fraction.

ATPase activity was measured by the continuous method of Pullman et al. (1960) and Fritz and Hamrich (1966) as reported by Koch (1971/72). A 1-ml reaction mixture contained components at the following concentrations: 4.5 mM ATP, 5 mM Mg^{2+}, 100 mM Na^+, 20 mM K^+, 135 mM imidazole buffer (pH 7.5), 0.19 mM NADH, 0.5 mM PEP, 0.02% BSA, approx. 9 units pyruvate kinase and 12 units lactic dehydrogenase, and 50 µl homogenate fraction (15-25 µg protein). Absorbance changes were measured at 340 nm for 10 min using a Gilford recording spectrophotometer with temperature controlled at 37°C in reaction mixture. Total ATPase activity was determined in the presence of Na^+, K^+ and Mg^{2+}. Mg^{2+} ATPase activity was measured using the same mixture plus ouabain at a concentration of 1.0 mM. Na^+-K^+ATPase activity is total activity minus Mg^{2+}ATPase activity. Oligomycin (2 µg/ml in the reaction mixture) was used to delineate oligomycin-sensitive (mitochondrial) and insensitive Mg^{2+}ATPases. The data obtained were based on the duplicate determinations. The variation in the replicate values was less than 5% of the average. Absorbance at 340 nm per 10 min per 10 µg protein at 37°C used for measurement of ATPase activities. Specific activity = µmoles Pi mg^{-1} protein hr^{-1}. Oligomycin used to determine mitochondrial Mg^{2+} ATPase (oligomycin sensitive) and oligomycin insensitive activities.

Pesticides used in inhibitor studies were added as ethanol (95%) solution. The solutions were prepared so that a 1 µl addition gave the desired pesticide concentration in the reaction mixture. The ethanol solution was injected into a rapidly stirring reaction mixture (using a Jr. Vortex mixer) with a Hamilton 1 µl microsyringe. Protein-Kel derivatives and antibody preparations were added to the reaction mixture using 10-100 µl microsyringes or micropipetters.

Results and Discussion

Initially the compounds related to Kepone, which were to be used in the covalent binding to BSA, were evaluated as inhibitors of the ATPase activities from a fire ant head homogenate fraction. Table 2 shows that Kelevan (the levulinate derivative of Kepone) was much less effective than Kepone or DCPD. However, Kelevan caused over 50% inhibition of mitochondrial Mg^{2+}ATPase activity at 6×10^{-5}M. A BSA-Kel preparation was also tested and found to cause inhibition of fire ant head ATPase activities (molar concentration of Kel in the BSA-Kel was estimated to be about 20 µM).

TABLE 2. COMPARISON OF SEVERAL COMPOUNDS RELATED TO KEPONE FOR THEIR INHIBITION OF ATPASE ACTIVITIES FROM A FIRE ANT HEAD HOMOGENATE FRACTION

Addition	Per Cent Inhibition		
	Na^+-K^+ ATPase	Mg^{2+} ATPase	
		Oligomycin	
		Sensitive	Insensitive
Kepone (15 μM)	73	100	25
Kelevan (60 μM)	31	52	42
KS* (20 μM)	75	82	21
DCPD (7.5 μM)	36	92	20

*KS - Kelevan N-hydroxysuccinimide.

Table 3 shows the results from the first test of a crude antiserum (used without fractionation or purification) compared to control antiserum. The antiserum had been shown to contain antibodies to Kelevan by the Micro-Ouchterlony immunodiffusion test (see Immunology Studies). It was clear from this test that the antiserum could prevent the inhibition of ATPase activities by DCPD (Table 3).

A large sample of antiserum was obtained and fractionated with sodium sulfate (see Materials and Methods). The immunoglobulin (Ig IV) fraction was tested for protection (Table 4, Ig added with DCPD before enzyme) and reversal (Table 5, Ig added 5 min after combining enzyme and Kepone) of ATPase activities inhibited by organochlorine pesticides.

TABLE 3. EFFECT OF RABBIT ANTISERUM TO BSA-KEL ON THE INHIBITION OF FIRE ANT HEAD HOMOGENATE ATPASE ACTIVITIES BY DCPD

Addition	Per Cent Inhibition		
	Na^+-K^+ ATPase	Mg^{2+} ATPase	
		Oligomycin	
		Sensitive	Insensitive
DCPD (7.5 µM)*	36	92	20
Antiserum (50 µl)*	26	27	+45
Antiserum (50 µl)** +DCPD (7.5 µM)	0	0	0
Control Serum* (50 µl)	15	0	+33
Control Serum***(50 µl) +DCPD (7.5 µM)	16	54	26

*Compared to control
**Compared to antiserum only activities
***Compared to control serum only activities

TABLE 4. EFFECT OF RABBIT ANTISERUM* TO BSA-KEL ON DCPD INHIBITION OF FAH (MS)

Additions	FAH (MS) ATPase Activities		
	Na^+-K^+	Mito	Oligo
ATP + Enz	.100	.041	.040
ATP + DCPD (7.5µm)→Enz	.003	.003	.046
ATP + Ig IV(1.2mg)→Enz	.009	.030	.032
ATP + DCPD→Ig IV→Enz	.084	.012	.028

*Antiserum was fractionated and the immunoglobulin fraction (IgIV) was used.

TABLE 5. EFFECT OF RABBIT ANTISERUM* ON KEPONE INHIBITION OF DOG BRAIN (NEP) ATPASE ACTIVITIES WITH SUBSTRATE ADDITION LAST

Additions	ATPase Activities		
	Na^+-K^+	Mito	Oligo
Enz+ATP	.126	.065	.108
Control Ig (1.2mg)+Enz(10')→ATP	.112	.074	.104
Ig IV (1.2mg)+Enz(10')→ATP	.119	.066	.092
Kepone (3.75μM)+Enz(10')→ATP	.102	.018	.087
Kepone (3.75μM)+Enz(5')→Control Ig(5')→ATP	.098	.033	.098
Kepone (3.75μM)+Enz(5')→Ig IV(5')→ATP	.120	.068	.089

*Antiserum was fractionated and Ig IV was used.

The results in Table 4 show that nearly complete reversal of inhibition by DCPD occurred for Na^+-K^+ATPase activity and that some but much less reversal of mitochondrial Mg^{2+}ATPase activity was observed. However, apparently complete protection was obtained against inhibition of ATPase activities by Kepone in a dog brain preparation (Table 5).

With apparent success in the production of a specific antibody to Kepone and DCPD additional synthesis of Kelevan derivatives of BSA, BGG and BF were carried out. As mentioned in the Chemistry Studies, these synthetic reactions were conducted in small batches because a poorer yield of derivative was obtained when large batch synthesis was attempted. Also because of the availability of chicken rearing facilities and the fact that chickens produce larger quantities of immunoglobulins, fifteen chickens were injected with BSA-Kel in complete Freund's adjuvant. As mentioned earlier, the chickens responded with a good production of antiserum as determined by micro-Ouchterlony test and affinity column isolation (Figure 3). Reversal of Kepone inhibition of ATPase activity by chicken produced antibody to Kepone was observed from dog brain (Table 5), mice heart (Table 6), and fly homogenate preparations (see last section of report).

TABLE 6. REVERSAL OF KEPONE INHIBITION OF MICE HEART MITOCHONDRIAL MG^{++}ATPASE ACTIVITY BY A PURIFIED ANTIBODY FROM CHICKEN

Additions	Antibody	ATPase Activities*	Inhibition %
Enzyme	--	10.97	--
+ Kepone**		5.98	
+ Control-GG (Crude)	2mg	10.47	0
+ Control-GG (Crude) + Kepone	2mg	5.90	46
+ Ab (Crude)	2mg	10.07	0
+ Ab (crude) + Kepone	2mg	10.10	0
+ Control-GG (Purified)	62ug	10.50	0
+ Control-GG (Purified) + Kepone	62ug	5.70	46
+ Ab (Purified)	62ug	10.47	0
+ Ab (Purified) + Kepone	62ug	8.98	18
+ Control-GG (Purified)	124ug	10.40	0
+ Control-GG (Purified) + Kepone	124ug	5.90	46
+ Ab (Purified)	124ug	10.70	0
+ Ab (Purified) + Kepone	124ug	9.98	9

Values are averages of 3 observations.
GG, γ-globulin; Ab, Antibody.
* μmoles Pi mg^{-1} protein hr^{-1}.
**Kepone Conc. 3×10^{-7}M.

Isolation of Pure Antibody by Affinity Chromatography

The Sepharose-BGG-Kel preparation (see Materials and Methods) was used to prepare a small affinity chromatography column (0.6 x 11 cm). Small samples (0.4 ml) of control serum and antiserum were tested for binding of Kepone reactive immunoglobulins. Figure 3 shows typical results obtained from a number

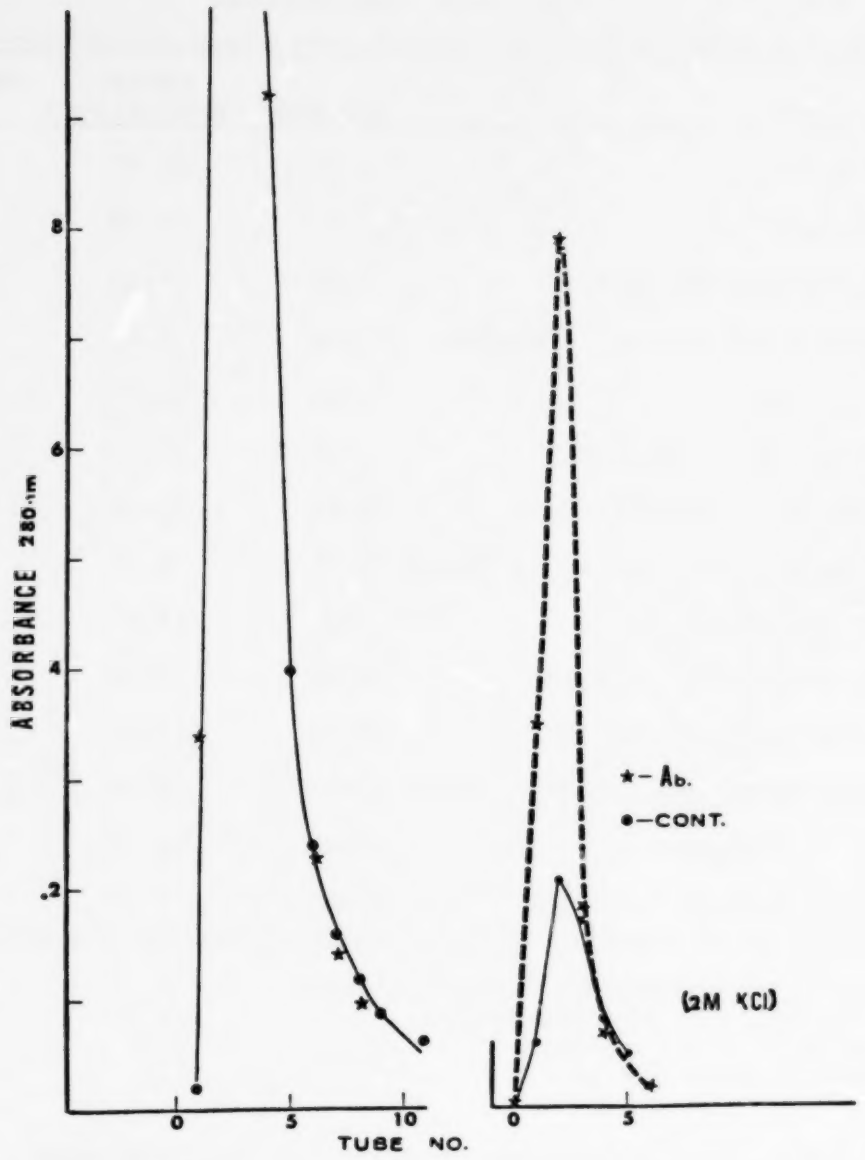

Figure 3. Elution patterns for control serum and BSA-Kelevan antiserum from chickens. Blood samples allowed to clot and sera added directly to a washed (Tris-Hcl, pH 7.0) BGG-Kel Sepharose 4B affinity column. Large peak (left) unbound protein. Peaks on right (0-control serum, *-antiserum, Ab.) eluted with 2M KCl in Tris-HCl, pH 7.0.

of such separations. Flow rate and fraction collection were set to collect 2 ml per tube per 5 min. The large peak (left curve Fig. 1) was the same for both blood serum samples (●-Control serum, *-antiserum) and represents unbound protein that was eluted with Tris-HCl buffer. The control serum samples always contained a small amount of protein that bound to the affinity column. However, the serum samples from BSA-Kel treated chickens which contained "active antiserum yielded much larger 280 nm absorption peaks." It was observed that the above small affinity column could determine the presence of antiserum for Kepone in about one hour. By comparison the micro-Ouchterlony test takes several days to indicate the presence of specific antibodies to BGG-Kel.

The larger affinity column (see Materials and Methods) was used to obtain sufficient quantity of the specific binding protein (antibodies to the hapten Kelevan in BSA-Kel) to determine its effect on Kepone inhibition of the oligomycin (mitochondrial) sensitive Mg^{2+} ATPase activity from a mice heart homogenate 13,000 x g fraction. (It was shown in other studies by Anwar Saad in the P.I.'s laboratory that the Mg^{2+}ATPase activity in mice heart preparations was particularly sensitive to Kepone.) Table 6 shows that BGG-Kel affinity column strong protection against Kepone inhibition of ATPase activity was observed at 124 µg protein per ml reaction mixture. Additional specific antibody preparations from the affinity column showed complete reversal of Kepone inhibition at less than 100 µg protein per ml reaction mixture (see last section).

A sample of the specific binding protein from the affinity column was analyzed by Dr. E. A. Lewis, Department of Chemistry, University of Alabama, Tuscaloosa, Alabama, by ultracentrifugation. The results of his analysis are shown in Figure 4. Two samples were analyzed: Sample A - the serum was fractionated by Na_2SO_4 and the partially purified Ig was passed through the affinity column; Sample B - the blood serum was applied directly to the affinity column. Dr. Lewis stated that there was no real difference in sedimentation coefficients (S) for A and B. Sample A was run at a higher concentration and probably accounts for the small increase in S. The boundary profiles for both samples were symmetrical and the samples must be homogeneous. Only one late time (typical) cell boundary region tracing is shown in Figure 4. The sedimentation coefficient (S = 5.79 ave.) is indicative of an immunoglobulin.

Effect of Antibody to Hapten (Kelevan) on Inhibition of ATPase Activities by Pesticides Inhibitory to the ATPase System

A study was conducted after the termination date of this grant (February 14, 1978) on the specificity of the antibody produced against the antigen BSA-Kel. A sufficient quantity of

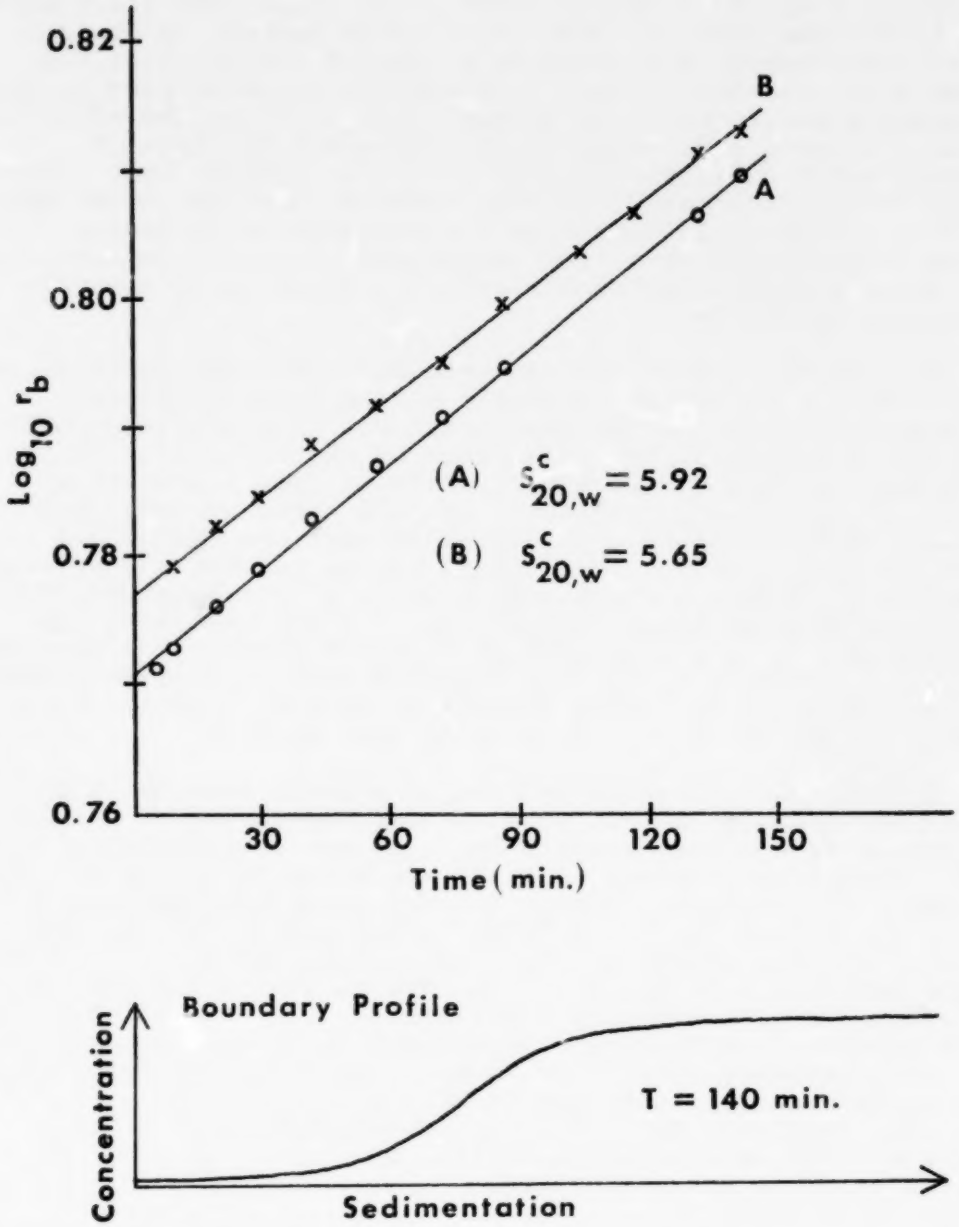

Figure 4. Ultracentrifugation analysis of specific binding protein (Kepone antibody) from BGG-Kel affinity column. Slope A and B = sedimentation coefficients. Boundary profile obtained by computer analysis of a series of boundary region tracings recorded over approximately 2.5 hr

purified antibody was not available prior to the termination date to conduct these comparison studies. The synthesis of the BGG-Kel affinity column and of good antibody production in 11 to 12 chickens in the last group of 15 chickens injected with BSA-Kel made it possible to conduct these studies.

The results of preliminary experiments were surprising and quite unexpected. It appears from the results that the antibody to Kepone was generally effective against organochlorine pesticides that inhibit the ATPase system. It was as effective in preventing and reversing in vitro inhibition of mitochondrial Mg^{2+}ATPase activity from rat heart by Kepone (1 µM) and DDT (2 µM). Inhibition of ATPase was also greatly reduced or prevented by the antibody (72 µg/ml reaction mixture) using reduced mirex (mixture of mono- and dihydro-winged derivatives) (5 µM), Kelthane (2 µM), and aldrin (20 µM). In the experiments with Kepone and DDT, reversal of ATPase inhibition was proportional to amount of antibody added. As little as 7.2 µg antibody protein in 1 ml reaction mixture reduced the inhibition by 1 µM Kepone and 2 µM DDT by nearly 40%. Inhibition by Kepone was completely prevented by 144 µg antibody. Inhibition by DDT was only 6% in the presence of 144 µg antibody.

The above results indicate that the antibody to Kepone produced by the antigen BSA-Kelevan has a rather broad specificity to the organochlorine portion of these pesticides. To determine this possibility, the protection by antibody of mito.-Mg^{2+}ATPase activity by Plictran and pyrethrin was investigated. The inhibition by Plictran was not affected by the presence of 72 µg antibody. However, the inhibition by pyrethrin appeared to be somewhat (20%) less in the presence of the antibody (72µg), but the protection was not nearly as great at that observed for the organochlorine pesticides. The latter observation with pyrethrin will require further study; however, it appears that the antibody produced by BSA-Kel has a broad specificity for organochlorine pesticide that inhibit the ATPase system.

Further investigation of this observation is being conducted. Concentration response studies are underway to determine binding affinity relationships between the purified antibody and the different organochlorine pesticides. The above findings, if proved correct, indicate that the binding site for the different organochlorine pesticide inhibitors on the ATPase complexes must be the same or very similar. If this should prove to be so, the reason for the difference in effectiveness of different organochlorine pesticides may be related to their ability to penetrate the lipoprotein complex of the enzyme system. Studies to prove this latter possibility will be greatly assisted by the availability of the antibody produced under partial support by this EPA Grant.

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